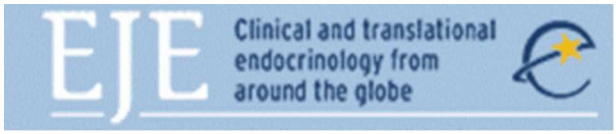
Title	Adiponectin gene variants and the risk of coronary heart disease: a 16-year longitudinal study
Author(s)	Cheung, CYY; Hui, YLE; Cheung, BMY; Woo, YC; Xu, A; Fong, HY; Ong, KL; Yeung, CY; Janus, ED; Tse, HF; Sham, PC; Lam, KS
Citation	European Journal of Endocrinology, 2014, v. 171, p. 107-115
Issued Date	2014
URL	http://hdl.handle.net/10722/203086
Rights	Creative Commons: Attribution 3.0 Hong Kong License



Adiponectin gene variants and the risk of coronary heart disease: a 16-year longitudinal study

Journal:	*European Journal of Endocrinology*
Manuscript ID:	EJE-14-0079.R1
mstype:	Clinical Study
Date Submitted by the Author:	n/a
Complete List of Authors:	Cheung, Chloe; The University of Hong Kong, Department of Medicine Hui, Elaine; The University of Hong Kong, Department of Medicine Cheung, Bernard; The University of Hong Kong, Department of Medicine Woo, YC; The University of Hong Kong, Department of Medicine Xu, Aiming; University of Hong Kong, Medicine Fong, Carol; The University of Hong Kong, Department of Medicine Ong, Kwok Leung; University of Hong Kong, Department of Medicine Yeung, CY; The University of Hong Kong, Department of Medicine Janus, Edward; University of Melbourne, Western Hospital, Department of Medicine Tse, Hung-Fat; University of Hong Kong, Medicine Sham, Pak; University of Hong Kong, Department of Psychiatry; University of Hong Kong, Genome Research Centre Lam, Karen; University of Hong Kong, Department of Medicine
Keywords:	Coronary heart disease, genetic variants

Adiponectin gene variants and the risk of coronary heart disease: a 16-year longitudinal study

Short title: *ADIPOQ* +276G>T predicts incident CHD

Chloe YY Cheung[1*], Elaine YL Hui[1,3*], Bernard MY Cheung[1], YC Woo[1], Aimin Xu[1,3], Carol HY Fong[1], KL Ong[1,5], CY Yeung[1], Edward D Janus[6], Hung-Fat Tse[1,3], Pak C Sham[2,4†], and Karen SL Lam[1,3†]

[1]Department of Medicine and [2] Department of Psychiatry, [3]Research Centre of Heart, Brain, Hormone and Healthy Aging, the [4]Centre for Genomic Sciences, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong; [5]Centre for Vascular Research, University of New South Wales, Sydney, NSW 2052 Australia; [6]Department of Medicine, Northwest Academic Centre, The University of Melbourne, Western Hospital, Melbourne, Australia

*CYY Cheung and EYL Hui contributed equally to this work and should be considered as co-first authors; [†]PC Sham and KSL Lam contributed equally to the supervision of this work and are co-corresponding authors.

Co-corresponding authors:

Professor Karen SL Lam, Department of Medicine, The University of Hong Kong , Queen Mary Hospital, 102 Pokfulam Road, Hong Kong; Tel:(852)-22553348; Fax:(852)-28162863; Email: ksllam@hku.hk;

Professor Pak C Sham, Department of Psychiatry, The University of Hong Kong , Queen Mary Hospital, 102 Pokfulam Road, Hong Kong; Tel:(852)-22554486; Fax: (852)-28551345; Email : pcsham@hku.hk.

Keywords: Coronary heart disease, Adiponectin, *ADIPOQ*, +276G>T

Total word count: 3442 words

Disclosure Statement: The authors have nothing to disclose.

Abstract (word count: 247)

Objective: Circulating adiponectin levels have been shown to be associated with risk of coronary heart disease (CHD). However, its primary role in protecting against the development of CHD remains controversial due to conflicting observations in prospective studies. To gain further insight into the primary role of adiponectin, our major objective was to investigate the relationship between single nucleotide polymorphisms (SNPs) of the adiponectin gene (*ADIPOQ*) and incident CHD in a population-based cohort with no CHD at baseline.

Design and Methods: We conducted a 16-year longitudinal study in 2196 subjects from the Hong Kong Cardiovascular Risk Factors Prevalence Study (CRISPS). During 33,862 person-years of follow-up, 184 subjects developed CHD (cumulative incidence rate = 5.4 per 1000 person-years). Nine *ADIPOQ* SNPs with potential functional relevance or shown to be associated with adiponectin levels and/or CHD were genotyped.

Results: Among the 9 *ADIPOQ* SNPs, +276G>T (rs1501299) was independently associated with incident CHD in men but not in women, even after adjustments for traditional cardiovascular risk factors ($P_{adjusted}$ = 5.5x10^{-3} to 0.023; Hazard ratio [HR] = 1.39 to 1.54). Furthermore, there was a significant association of the T allele of +276G>T with lower adiponectin level (P = 0.027; β[95%CI]

54 = -0.05[-0.10, -0.01]).

55

56 **Conclusions:** This study demonstrated that +276G>T may be an independent predictor of CHD

57 development. Our findings suggest that low adiponectin levels, as may be influenced by +276G>T,

58 confer a higher risk of CHD, in keeping with a role of hypoadiponectinaemia in the development of

59 CHD in the general population.

60

61

62

63

Introduction

Adiponectin is one of the most abundant insulin-sensitising adipokines secreted by the adipocytes (1). Circulating levels of adiponectin are reduced in obesity, in particular visceral obesity (2, 3), and predispose to endothelial dysfunction, atherosclerosis and subsequent cardiovascular diseases in animal models. Hypoadiponectinaemia has been proposed as one of the mediators of increased cardiovascular risk in obesity (4). Adiponectin, encoded by *ADIPOQ,* is suggested to play a protective role in the development of coronary heart disease (CHD) due to its anti-inflammatory, anti-oxidative, anti-apoptotic, and anti-atherogenic properties (3, 5). The prospective relationship between circulating adiponectin level and the developement of CHD has been extensively investigated. However, whether adiponectin levels are causally related to CHD development remains controversial. While high adiponectin levels are believed to be protective against CHD in healthy populations (6), more recent studies suggest that high adiponectin levels are linked with a greater risk of CHD or cardiovascular mortality in older populations (7) or cohorts with prevalent CHD (8). As the up-regulation of adiponectin could act as a compensatory mechanism to limit further vascular injury (8), a high adiponectin level in the established disease state might reflect on the severity of the underlying vascular inflammation, and hence a positive association between the adiponectin level and cardiovascular mortality.

Studies of potentially functional *ADIPOQ* genetic variants may provide more insight into the

primary role of adiponectin, if any, in protecting against the development of CHD. So far, only a few prospective studies conducted in Caucasians have investigated the genetic effects of *ADIPOQ* SNPs on CHD development (9, 10, 11). The primary objective of this study was to evaluate the impact of 9 *ADIPOQ* single nucleotide polymorphisms (SNPs) on the risk of CHD in a 16-year longitudinal study cohort of healthy Southern Chinese. These SNPs were selected because of their potential functional relevance or reported influence on the risk of CHD or cardiovascular diseases (CVD) and/or adiponectin levels.

Methods

Subjects

The Hong Kong Cardiovascular Risk Factors Prevalence Study (CRISPS) is a population-based prospective study of cardiovascular risk factors in Hong Kong (12). In 1995-1996 (CRISPS1), 2895 Hong Kong Chinese were selected randomly by their telephone numbers to undergo a comprehensive assessment of cardiovascular risks. Subjects were contacted for reassessment in 2000-2004 (CRISPS2) and in 2005-2008 (CRISPS3). The latest CRISPS4 follow-up assessment was commenced in July 2010. The current study involved a total of 2196 subjects who did not have a history of CHD at baseline (CRISPS1) and with DNA samples available for genetic analysis. During 33,862 person-years of follow-up, 184 subjects (111 men and 73 women) who were non-CHD at baseline had developed CHD by the end of 2011, giving a cumulative incidence rate of 5.4 per 1000 person-years. 2012

subjects (915 men and 1097 women) who were non-CHD at baseline had remained as non-CHD at subsequent follow-up visit(s). CHD events were defined based on ICD-9 (402, 404, 410-414, 425-429) which included, among others, acute myocardial infarction (MI), heart failure, and angina pectoris as described in our previous study (13). Information on the dates of the CHD events and discharge diagnosis were obtained from the patients and also verified from the Hospital Authority database or the patients' private practitioners. For those who had died, causes and dates of death were determined from the Hong Kong Death Registry database. Two physicians reviewed the medical diagnoses independently; disagreements between them were resolved by a third physician. The concordance between the two physicians was 0.98. Written informed consent was obtained from each participant and the study protocol was approved by the Ethics Committee of the University of Hong Kong.

Anthropometric and biochemical measurements

Anthropometric (including body mass index [BMI], waist circumference (WC), systolic blood pressure [SBP] and diastolic blood pressure [DBP]) and biochemical parameters (including fasting plasma glucose [FPG], 2-h post-OGTT glucose [2hrG], triglyceride [TG], high-density lipoprotein cholesterol [HDL-C]; low-density lipoprotein cholesterol [LDL-C] and total cholesterol [TC]) were measured as previously described (14). Type 2 Diabetes (DM) was defined as FPG $\geq$ 7.0mmol/l or 2hrG $\geq$ 11.1mmol/l or both, according to the World Health Organization 1998 diagnostic criteria (15); or on anti-diabetic medication. At baseline, 181 subjects (89 men and 90 women) had DM and 1923

subjects (904 men and 1017 women) were non-DM. The presence of hypertension (HT) was defined as BP ≥ 140/90mmHg or receiving regular anti-hypertensive treatment. The presence of dyslipidaemia was defined as fasting TG ≥ 1.69mmol/l, HDL-C < 1.04mmol/l in male and < 1.29mmol/l in female), and LDL-C ≥ 3.4mmol/l (16), or taking lipid-lowering agents. Since stored baseline plasma samples were no longer available from a large number of subjects, total adiponectin level was measured in available plasma samples collected at the CRISPS2 follow-up visit (n=1676), using an in-house sandwich ELISA kit established in our laboratory (intra-assay and inter-assay coefficients of variation of 6.2–8.3% and 5.1–6.4% respectively) (17).

Genetic analysis

A total of 9 *ADIPOQ* SNPs (rs16861194, rs266729 [-11377C>G], -10677C>T, rs1802052 [-10066G>A], rs822395 [-4034A>C], rs822396 [-3964A>G], rs12495941, rs2241766 [+45T>G], and rs1501299 [+276G>T]) were selected on the basis of previous publications suggesting them as functional (18, 19, 20), or shown to affect adiponectin levels (9, 21, 22, 23), or associated with CHD/CVD (9, 11, 24). Genotyping of these SNPs was performed using the Sequenom iPLEX Gold genotyping assay at the Centre for Genomic Sciences, the University of Hong Kong. The average genotyping call rate and concordance rate were 99.8% and 97.5%, respectively. The SNPs were tested for deviation from Hardy Weinberg Equilibrium (HWE) by the De Finetti program available online at <http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl>.

Statistical Analysis

All statistical analyses were conducted with SPSS version 19.0 (Chicago, Illinois). The associations of SNPs with incident CHD were evaluated using Cox proportional hazards regression (Cox-regression) analyses under the additive model. Survival was calculated from the date of visit at baseline to the date of diagnosis of CHD or the date of last follow-up visit. In view of the known gender difference in adiponectin levels (25) and the observation of a higher incidence rate of CHD in men (7.1 per 1000 person years versus 4.0 per 1000 person years in women), we also performed gender-stratified analyses. A two-tailed $P < 0.05$ was considered as statistically significant. Baseline clinical parameters that were biologically likely to have an influence on the development of CHD or were statistically different ($P < 0.05$) between the incident CHD and non-CHD groups were adjusted for in the multiple adjustment analyses. Three different sets of traditional risk factors were included in the multiple Cox-regression analyses. Multiple adjustments for age, BMI, 2hrG, HOMA-IR, HDL-C, LDL-C, TG, SBP, DBP and smoking were made in Model 1; adjustments were made for age, BMI, FPG, 2hrG, HDL-C, LDL-C, TG, SBP, DBP and smoking in Model 2; and adjustments were made for age, BMI, DM, dyslipidaemia, HT and smoking in Model 3. Logistic regression analysis was employed to examine the association of +276G>T with DM at baseline. The association of +276G>T with plasma adiponectin level at CRISPS2 was evaluated by linear regression analysis. The associations of CRISPS2 adiponectin levels with the development of CHD were evaluated using

Cox-regression analyses. Survival was calculated from the date of visit at CRISPS2 to the date of diagnosis of CHD or the date of last follow-up visit. Both combined and gender-stratified analyses were performed. The study power was calculated using the Genetic Power Calculator (26).

Results

Baseline clinical characteristics

We examined the associations of 9 *ADIPOQ* genetic variants with incident CHD in 2196 subjects with DNA samples available for genetic analysis. Among these subjects who were non-CHD at baseline, 184 subjects (cumulative incidence rate = 5.4 per 1000 person years) developed CHD during 33,862 person-years of follow-up; 2012 subjects remained free of CHD at subsequent follow-up visit(s). Table 1 show the baseline clinical characteristics of the subjects. In both genders, the incident CHD groups had worse traditional cardiovascular risk factors, such as the presence of DM, dyslipidaemia and HT, when compared with the non-CHD groups. However there were no significant differences in regular exercise, alcohol drinking, and family history of DM, HT and CHD.

Association with CHD development

The allele frequencies of the 9 SNPs were comparable to those reported in HapMap or 1000 Genome Project (Table 2). The genotype distributions of these SNPs were in HWE (P = 0.071-0.809). Among these SNPs, rs1501299 (+276G>T) showed a significant association with incident CHD

($P_{unadjusted}$ = 0.042; HR[95%CI]: 1.26[1.01-1.56]). When stratified by gender, the association of +276G>T variant with incident CHD remained significant in men ($P_{unadjusted}$ = 0.020; HR[95%CI]: 1.39[1.05-1.83]). However, no significant association was found in women ($P_{unadjusted}$ = 0.816; HR[95%CI]: 1.04[0.73-1.50]). No significant associations of the other SNPs with incident CHD were observed in either gender.

Independent association of +276G>T with incident CHD in men

The possible independent association of +276G>T with incident CHD in men was further analysed with adjustments for different sets of traditional risk factors, including DM and its related traits, as the +276G>T variant was independently associated with DM at baseline, even after adjustment for age, sex and BMI in this study ($P_{age,\ sex\ and\ BMI\text{-}adjusted}$ = $2x10^{-3}$, OR[95%CI]: 1.45[1.14-1.84]). Table 3 shows the results of the multiple Cox-regression analyses in the male subjects. +276G>T showed a significant association with incident CHD in men after adjustment for age, BMI, 2hrG, HOMA-IR, HDL-C, LDL-C, TG, SBP, DBP and smoking (Model 1) ($P_{adjusted}$ = $5.5x10^{-3}$; HR[95%CI]: 1.54[1.13-2.08]). Figure 1 shows the cumulative survival curves for incident CHD in men, based on multiple adjustment model 1 and stratified by the +276G>T genotypes. The male subjects with the TT genotype had significantly higher risk of developing CHD than those with the GG or GT genotypes as shown in Figure 1 ($P_{adjusted}$ = $5.5x10^{-3}$). The association was also significant in adjustment Model 2 ($P_{age,\ BMI,\ FPG,\ 2hrG,\ HDL\text{-}C,\ LDL\text{-}C,\ TG,\ SBP,\ DBP\ and\ smoking\ adjusted}$ = 0.015;

HR[95%CI]: 1.44[1.08 -1.93]). Furthermore, when the presence of DM, dyslipidaemia and HT were included in the adjustment model in addition to age, BMI, and smoking (Model 3), the association persisted ($P_{adjusted}$ = 0.023; HR[95%CI]: 1.39[1.05-1.84]). In contrast, as expected, no significant association of +276G>T with incident CHD was observed in women (Model 1: $P_{adjusted}$ = 0.539; HR[95%CI]: 0.88[0.58-1.33]; Model 2: $P_{adjusted}$ = 0.381; HR[95%CI]: 0.83[0.56-1.13]; Model 3: $P_{adjusted}$ = 0.339; HR[95%CI]: 0.84[0.58-1.21]).

Association of the +276G>T variant with plasma adiponectin level at CRISPS2

Adiponectin level was found to be significantly lower (P<0.001) in men (median[interquartile range]: 5.62[3.62-8.69]mg/l, n=804) than in women (7.89[5.37-11.78]mg/l; n=872). We observed a significant association of the T allele of +276G>T with lower adiponectin level (P = 0.027; β[95%CI] = -0.05[-0.10, -0.01]) in 1676 subjects with available plasma samples. When the association was examined in men and women separately, we observed a significant association in men (P = 0.049; β[95%CI] = -0.07[-0.14, 0.00]), but the association was not significant in women (P = 0.424; β[95%CI] = -0.03[-0.09, 0.04]). Supplementary table 1 shows the comparison of adiponectin levels at CRISPS2 between different genotypes of +276G>T. Similar findings were obtained when subjects who had developed CHD by CRISPS2 were excluded.

Association of plasma adiponectin level at CRISPS2 with the development of CHD after a

median interval of ~9.6 years

We further examined the association of plasma adiponectin levels at CRISPS2 with the development of CHD, after a median interval of ~9.6 years. Since 46 of the 1676 subjects with adiponectin levels available for analysis had developed CHD by CRISPS2, only 1630 subjects were included in the analysis. Over a median interval of ~9.6 years, 101 subjects (56 men and 45 women) had developed CHD, while 1529 subjects (720 men and 809 women) remained free of CHD. We did not observe a significant association between CRISPS2 adiponectin level and CHD development, in both the combined (P = 0.200; HR[95%CI] = 0.82[0.60-1.11]) and gender-stratified analyses (Men: P = 0.465; HR[95%CI] = 0.85[0.60-1.30]; Women: P = 0.665; HR[95%CI] = 0.89[0.54-1.48]).

Discussion

In this study, we observed a significant association of +276G>T with CHD development in men in a general population, independent of conventional cardiovascular risk factors. Consistent with previous cross-sectional studies (27, 28, 29), the +276G>T variant showed a significant association with DM in our cohort. The current study demonstrated that the +276G>T variant was independently associated with incident CHD in men, even after adjustment for DM or its related traits, together with other potential confounding factors, in the different multiple adjustment models. We also observed that +276G>T was associated with lower plasma adiponectin in this Chinese population, as was previously reported in studies amongst Italians (21) and Greeks (23). Our findings suggest that low

adiponectin levels, as influenced by a genetic variant in the *ADIPOQ* gene, confer a higher risk of CHD, in keeping with a role of low circulating adiponectin levels in the development of CHD.

The T allele of +276G>T was previously found to be associated with CHD in case-control cross-sectional studies amongst Chinese (24), Italians (21) and Greeks (23). The current study has further provided evidence for its association with an increased risk of developing CHD in a population-based cohort, likely through a reduction in adiponectin expression. Intriguingly, a significant association of the +276G>T variant with incident CHD was only present in the male subjects of our cohort. The differences in cardiovascular risk profile between the two genders, such as lipid levels and blood pressure, may have contributed to the observed gender-specific association. The higher CHD incidence rate in men (7.1 per 1000 person years) compared to women (4.0 per 1000 person years), may also be a contributing factor. The lack of a significant association in women might have been attributable to the smaller number of new CHD event. Adiponectin levels were known to be higher in women and decline with abdominal adiposity, which also show gender-specific differences (25, 30). Indeed, adiponectin level was found to be higher in the female subjects of our cohort. Gender-specific differences in adiponectin levels have been shown to be strongly associated with serum androgen levels (25). Our group has previously demonstrated that testosterone selectively decreased the circulating concentrations of the high molecular weight form of adiponectin by impeding its secretion from the adipose tissue (17). Androgens have been shown to decrease plasma

adiponectin and the androgen-induced hypoadiponectinaemia may lead to higher risk of atherosclerosis in men (31). The unfavourable consequences of lower adiponectin levels could possibly lead to a higher risk of developing CHD in men than in women. Therefore, the genetic effect of *ADIPOQ* might be more readily detected in the high risk male subjects.

We have demonstrated, in this long-term longitudinal study, the independent association of *ADIPOQ* +276G>T with incident CHD in a general population. In previous prospective studies which examined the genetic effect of +276G>T on CHD (11) or CVD (9, 10) development, a US study based on diabetic men reported a significant association of this SNP with CVD development (9). Contrary to our findings, they reported that the T allele was associated with a lower risk of CVD and increased adiponectin level (9). The discrepancy may be explained by the difference in inclusion criteria (subjects from the general population versus all DM patients and study endpoints (CHD versus CVD). Two other longitudinal studies (10, 11) did not detect a significant association of +276G>T, but reported significant associations of rs266729 and rs17300539 (monomorphic in Han Chinese), with CVD development (10); and rs822395 with CHD risk (11). However, no significant associations of rs266729 and rs822395 were detected in this study. Ethnic differences in genetic composition and interaction, together with distinct environmental factors, may contribute to these variations in findings.

The +276G>T variant was located within intron 2 of *ADIPOQ*. The biological significance of this SNP and the mechanism of which this variant leads to altered adiponectin levels has not been fully elucidated. We postulated that it may potentially affect the transcriptional activity or the splicing efficiency of the *ADIPOQ* gene. Previous studies have demonstrated that intronic polymorphisms can influence the transcription activity or splicing processes, even when the variants are located more than 30bp away from the nearest splice junction (32). Intronic splicing control elements, which are involved in recognition of the appropriate splice site or in regulation of the splice site usage, have been found as far as 200bp away from the splice site (33). Therefore, the +276G>T variant, which is located ~60bp away from the nearest splice junction, may potentially affect the splicing efficiency. Future functional studies would be helpful to delineate the effect of this variant on adiponectin protein expression.

This study has several limitations. The relatively small number of incident CHD cases, despite the long follow-up period, has made this study slightly underpowered. Nonetheless, the current sample size could achieve over 80% power to detect a significant association of +276G>T with a large effect size of 1.54 in men, at a significance level of 0.05. On the other hand, the effects of the other *ADIPOQ* SNPs may be too modest for a significant association to be detected. This study was limited by the small number of hard endpoint cases, such as MI, and has therefore made it difficult for a definitive conclusion to be drawn. Furthermore, the current study would have been significantly

strengthened with the detailed analyses of more CHD phenotypes. However, as data were retrieved from the Hospital Authority database, detailed information on coronary disease severity, such as the degree of coronary stenosis, were not available. Due to the limited plasma samples available for the analysis of adiponectin levels at baseline, adiponectin levels obtained at CRISPS2 were used as an alternative to examine the association with the +276G>T variant and incident CHD in the current study. However, with the small sample size, in particular the limited number of incident CHD cases, as well as a shorter follow-up interval of ~9.6 year, we were unable to demonstrate a significant association between CRISPS2 adiponectin level and the development of CHD. Nevertheless, the HRs were in keeping with the protective effect of adiponectin against CHD development in a general population initially free of the disease. Furthermore, we have only considered most, but not all, confounding factors for the development of CHD. Due to the observational study design, detailed information on lifestyle modification or treatment interventions, including the effects of different drug used and changing dosage during the study period, which may act as potential confounding factors, were not available for analyses. Taken into consideration these potential biases, effects of dysglycaemia (including DM, FPG and 2hrG), hypertension and dyslipidaemia were adjusted for in the multiple adjustment analyses. Other confounding factors in this observational study, such as the effect of attrition, may also lead to potential bias. The CRISPS cohort is a population-based study of the Hong Kong Southern Chinese. The findings from this study may not be generalised to other nations. Nonetheless, our data represents one of the largest longitudinal cohorts of Chinese subjects

with a long follow-up period. With the growing epidemic of CHD risk factors, such as DM and hyperlipidaemia, in the Chinese population, the present findings may still be clinically significant. Further prospective studies in Chinese, as well as in other populations would be useful to validate our results.

In conclusion, this study demonstrated that the +276G>T variant of *ADIPOQ*, associated with low circulating adiponectin levels, may be an independent predictor of CHD in community-based Southern Chinese men initially free of CHD. The current study has provided evidence that low adiponectin level, as may be affected by a genetic variant in the *ADIPOQ* gene, confer a greater risk of CHD. Our data are suggestive of a protective role of adiponectin in CHD development in the healthy population. Our findings also support the notion that high adiponectin levels are associated with a lower risk of CHD in healthy populations, whereas high levels in established cardiovascular disease may reflect a compensatory up-regulation of adiponectin. The +276G>T variant of *ADIPOQ* may affect the adiponectin gene expression and may act as a potential genetic marker for the prediction of CHD.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Hong Kong Research Grant Council (RGC) Theme-Based Research Scheme (TBRS) Grant T12-705/11.

Author contributions

CYYC, EYLH, KSLL and PCS conceived and designed the experiments. KSLL initiated and supervised the study. CYYC performed the experiments and analyzed the data. CYYC and EYLH wrote the paper. AX, YCW, CYY, KLO and CHYF collected the data and provided advice on experiments and data analysis. KSLL, PCS, BMYC, EDJ and HFT critically revised the manuscript.

Acknowledgements

We would like to thank all the participants of CRISPS and the medical staff of the Department of Health, Hong Kong SAR, for their assistance with data retrieval on life-death status and causes of death.

346 **References**

347

348 1. Hui X, Lam KS, Vanhoutte PM & Xu A. Adiponectin and cardiovascular health: an update.

349 *Br J Pharmacol* 2012 **165** 574-590.

350 2. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I,

351 Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K,

352 Muraguchi M, Ohmoto Y, Funahashi T & Matsuzawa Y. Paradoxical decrease of an

353 adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 1999 **257**

354 79-83.

355 3. Lam KS & Xu A. Adiponectin: protection of the endothelium. *Curr Diab Rep* 2005 **5**

356 254-259.

357 4. Shargorodsky M, Boaz M, Goldberg Y, Matas Z, Gavish D, Fux A & Wolfson N.

358 Adiponectin and vascular properties in obese patients: is it a novel biomarker of early

359 atherosclerosis? *Int J Obes (Lond)* 2009 **33** 553-558.

360 5. Yamauchi T, Kamon J, Waki H, Imai Y, Shimozawa N, Hioki K, Uchida S, Ito Y, Takakuwa

361 K, Matsui J, Takata M, Eto K, Terauchi Y, Komeda K, Tsunoda M, Murakami K, Ohnishi Y,

362 Naitoh T, Yamamura K, Ueyama Y, Froguel P, Kimura S, Nagai R & Kadowaki T. Globular

363 adiponectin protected ob/ob mice from diabetes and ApoE-deficient mice from

364 atherosclerosis. *J Biol Chem* 2003 **278** 2461-2468.

365 6. Koenig W, Khuseyinova N, Baumert J, Meisinger C & Lowel H. Serum concentrations of

adiponectin and risk of type 2 diabetes mellitus and coronary heart disease in apparently healthy middle-aged men: results from the 18-year follow-up of a large cohort from southern Germany. *J Am Coll Cardiol* 2006 **48** 1369-1377.

7. Poehls J, Wassel CL, Harris TB, Havel PJ, Swarbrick MM, Cummings SR, Newman AB, Satterfield S & Kanaya AM. Association of adiponectin with mortality in older adults: the Health, Aging, and Body Composition Study. *Diabetologia* 2009 **52** 591-595.

8. Wilson SR, Sabatine MS, Wiviott SD, Ray KK, De Lemos JA, Zhou S, Rifai N, Cannon CP & Morrow DA. Assessment of adiponectin and the risk of recurrent cardiovascular events in patients presenting with an acute coronary syndrome: observations from the Pravastatin Or atorVastatin Evaluation and Infection Trial-Thrombolysis in Myocardial Infarction 22 (PROVE IT-TIMI 22). *Am Heart J* 2011 **161** 1147-1155 e1141.

9. Qi L, Li T, Rimm E, Zhang C, Rifai N, Hunter D, Doria A & Hu FB. The +276 polymorphism of the APM1 gene, plasma adiponectin concentration, and cardiovascular risk in diabetic men. *Diabetes* 2005 **54** 1607-1610.

10. Gable DR, Matin J, Whittall R, Cakmak H, Li KW, Cooper J, Miller GJ & Humphries SE. Common adiponectin gene variants show different effects on risk of cardiovascular disease and type 2 diabetes in European subjects. *Ann Hum Genet* 2007 **71** 453-466.

11. Pischon T, Pai JK, Manson JE, Hu FB, Rexrode KM, Hunter D & Rimm EB. Single nucleotide polymorphisms at the adiponectin locus and risk of coronary heart disease in men

and women. *Obesity (Silver Spring)* 2007 **15** 2051-2060.

12. Janus ED. Epidemiology of cardiovascular risk factors in Hong Kong. *Clin Exp Pharmacol Physiol* 1997 **24** 987-988.

13. Chow WS, Tso AW, Xu A, Yuen MM, Fong CH, Lam TH, Lo SV, Tse HF, Woo YC, Yeung CY, Cheung BM & Lam KS. Elevated circulating adipocyte-fatty acid binding protein levels predict incident cardiovascular events in a community-based cohort: a 12-year prospective study. *J Am Heart Assoc* 2013 **2** e004176.

14. Cheung BM, Wat NM, Man YB, Tam S, Thomas GN, Leung GM, Cheng CH, Woo J, Janus ED, Lau CP, Lam TH & Lam KS. Development of diabetes in Chinese with the metabolic syndrome: a 6-year prospective study. *Diabetes Care* 2007 **30** 1430-1436.

15. Alberti KG & Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998 **15** 539-553.

16. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA* 2001 **285** 2486-2497.

17. Xu A, Chan KW, Hoo RL, Wang Y, Tan KC, Zhang J, Chen B, Lam MC, Tse C, Cooper GJ & Lam KS. Testosterone selectively reduces the high molecular weight form of adiponectin

by inhibiting its secretion from adipocytes. *J Biol Chem* 2005 **280** 18073-18080.

18. Barth N, Langmann T, Scholmerich J, Schmitz G & Schaffler A. Identification of regulatory elements in the human adipose most abundant gene transcript-1 (apM-1) promoter: role of SP1/SP3 and TNF-alpha as regulatory pathways. *Diabetologia* 2002 **45** 1425-1433.

19. Zhang D, Ma J, Brismar K, Efendic S & Gu HF. A single nucleotide polymorphism alters the sequence of SP1 binding site in the adiponectin promoter region and is associated with diabetic nephropathy among type 1 diabetic patients in the Genetics of Kidneys in Diabetes Study. *J Diabetes Complications* 2009 **23** 265-272.

20. Laumen H, Saningong AD, Heid IM, Hess J, Herder C, Claussnitzer M, Baumert J, Lamina C, Rathmann W, Sedlmeier EM, Klopp N, Thorand B, Wichmann HE, Illig T & Hauner H. Functional characterization of promoter variants of the adiponectin gene complemented by epidemiological data. *Diabetes* 2009 **58** 984-991.

21. Filippi E, Sentinelli F, Romeo S, Arca M, Berni A, Tiberti C, Verrienti A, Fanelli M, Fallarino M, Sorropago G & Baroni MG. The adiponectin gene SNP+276G>T associates with early-onset coronary artery disease and with lower levels of adiponectin in younger coronary artery disease patients (age <or=50 years). *J Mol Med (Berl)* 2005 **83** 711-719.

22. Ong KL, Li M, Tso AW, Xu A, Cherny SS, Sham PC, Tse HF, Lam TH, Cheung BM & Lam KS. Association of genetic variants in the adiponectin gene with adiponectin level and hypertension in Hong Kong Chinese. *Eur J Endocrinol* 2010 **163** 251-257.

23. Antonopoulos AS, Tousoulis D, Antoniades C, Miliou A, Hatzis G, Papageorgiou N, Demosthenous M, Tentolouris C & Stefanadis C. Genetic variability on adiponectin gene affects myocardial infarction risk: The role of endothelial dysfunction. *Int J Cardiol* 2012 **168** 326-330.

24. Gui MH, Li X, Jiang SF, Gao J, Lu DR & Gao X. Association of the adiponectin gene rs1501299 G>T variant, serum adiponectin levels, and the risk of coronary artery disease in a Chinese population. *Diabetes Res Clin Pract* 2012 **97** 499-504.

25. Bottner A, Kratzsch J, Muller G, Kapellen TM, Bluher S, Keller E, Bluher M & Kiess W. Gender differences of adiponectin levels develop during the progression of puberty and are related to serum androgen levels. *J Clin Endocrinol Metab* 2004 **89** 4053-4061.

26. Purcell S, Cherny SS & Sham PC. Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. *Bioinformatics* 2003 **19** 149-150.

27. Hara K, Boutin P, Mori Y, Tobe K, Dina C, Yasuda K, Yamauchi T, Otabe S, Okada T, Eto K, Kadowaki H, Hagura R, Akanuma Y, Yazaki Y, Nagai R, Taniyama M, Matsubara K, Yoda M, Nakano Y, Tomita M, Kimura S, Ito C, Froguel P & Kadowaki T. Genetic variation in the gene encoding adiponectin is associated with an increased risk of type 2 diabetes in the Japanese population. *Diabetes* 2002 **51** 536-540.

28. Ramya K, Ayyappa KA, Ghosh S, Mohan V & Radha V. Genetic association of ADIPOQ gene variants with type 2 diabetes, obesity and serum adiponectin levels in south Indian

population. *Gene* 2013 **532** 253-262.

29. Arikoglu H, Ozdemir H, Kaya DE, Ipekci SH, Arslan A, Kayis SA & Gonen MS. The Adiponectin variants contribute to the genetic background of type 2 diabetes in Turkish population. *Gene* 2014 **534** 10-16.

30. Matsuzawa Y, Funahashi T, Kihara S & Shimomura I. Adiponectin and metabolic syndrome. *Arterioscler Thromb Vasc Biol* 2004 **24** 29-33.

31. Nishizawa H, Shimomura I, Kishida K, Maeda N, Kuriyama H, Nagaretani H, Matsuda M, Kondo H, Furuyama N, Kihara S, Nakamura T, Tochino Y, Funahashi T & Matsuzawa Y. Androgens decrease plasma adiponectin, an insulin-sensitizing adipocyte-derived protein. *Diabetes* 2002 **51** 2734-2741.

32. Cooper DN. Functional intronic polymorphisms: Buried treasure awaiting discovery within our genes. *Hum Genomics* 2010 **4** 284-288.

33. Majewski J & Ott J. Distribution and characterization of regulatory elements in the human genome. *Genome Res* 2002 **12** 1827-1836.

457 **Figure legend**

458

459 Figure 1. The cumulative survival curves for incident CHD in men, based on multiple Cox

460 regression model 1, with adjustment for age, BMI, 2hrG, HOMA-IR, HDL-C, LDL-C, TG, SBP,

461 DBP and smoking. The cumulative survival curves were stratified by the +276G>T genotypes.

Table 1. Baseline clinical characteristics of subjects.

	All		Male		Female	
Baseline Parameters	Non-CHD	Incident CHD	Non-CHD	Incident CHD	Non-CHD	Incident CHD
n	2012	184	915	111	1097	73
Sex (Male %)	45.5	60.3**	-	-	-	-
Age (year)	44.4±11.7	56.3±10.9**	44.9±12.3	55.6±10.8**	44.0±11.2	57.2±11.0**
BMI (kg/m^2)	24.0±3.6	25.8±3.3**	24.3±3.3	25.8±3.3**	23.9±3.7	25.8±3.3**
Waist circumference (cm)	M: 82.6±9.1 F: 74.8±9.0	M: 88.1±9.5** F: 82.5±8.4**	82.5±9.1	88.1±9.5**	74.8±9.0	82.5±8.4**
Fasting glucose (mmol/l)	5.3±1.1	6.1±2.3*	5.4±1.3	5.8±1.8*	5.2±0.9	6.6±2.9*
2hr post-OGTT glucose (mmol/l)	6.6±2.7	8.5±5.0**	6.5±3.1	7.9±4.4**	6.7±2.4	9.4±5.9**
DM (%)	7.1	24.5**	7.8	18.9**	6.5	32.9**
DM Family history (%)[a]	17.4	13.7	15.9	9.2	18.6	20.5
Fasting insulin (μU/ml)[b]	4.8(3.1-7.3)	5.7(3.9-9.0) **	3.0(2.0-4.6)	3.7(2.6-5.3)*	3.3(2.3-5.0)	5.1(3.1-6.9)**
HOMA-IR[a]	1.1(0.7-1.7)	1.5(1.0-2.3) **	0.7(0.4-1.1)	0.9(0.6-1.3)*	0.7(0.5-1.1)	1.2(0.7-1.8)**
Dyslipidemia (%)	62.4	82.1**	61.9	79.3**	62.9	86.3**
TC (mmol/l)	5.0±1.0	5.4±1.0**	5.1±1.1	5.3±0.9**	4.9±1.0	5.6±1.1**
HDL (mmol/l)	1.3±0.3	1.2±0.3**	1.2±0.3	1.1±0.3*	1.4±0.3	1.3±0.3*
LDL (mmol/l)	3.2±0.9	3.6±1.0**	3.3±0.8	3.5±0.8	3.1±0.9	3.7±1.1**
TG[b] (mmol/l)	1.0(0.7-1.4)	1.3(0.9-1.9) *	1.1(0.8-1.6)	1.4(1.0-2.0)*	0.6(0.5-0.9)	0.9(0.6-1.1)*
SBP[c] (mmHg)	117.6±18.9	135.1±23.3**	120.0±17.0	133.3±21.2**	115.5±20.0	137.8±25.9**
DBP[d] (mmHg)	74.1±10.7	81.2±11.9**	76.6±10.0	81.6±12.4**	72.0±10.9	80.5±11.0**
HT (%)	14.1	48.4**	14.8	45.9**	13.6	52.1**

HT Family history (%)[e]	29.4	24.0	28.7	20.9	30.1	28.8
Taking anti-hypertensive drug	3.2	12.4**	3.2	8.8*	3.3	17.9**
Regular exercise (%)	41.5	44.6	44.8	47.7	38.7	39.7
CHD Family history (%)	10.4	13.8	10.9	5.5	16.2	17.8
Smoking (%)	22.6	37.2**	45.5	56.8*	3.5	6.9
Alcohol drinking (%)	36.1	42.9	56.1	59.1	19.4	18.1

**P-value < 0.001; *P-value < 0.05; Data as mean ± standard deviation or median with interquartile range. [a]Diabetes in first degree relatives. [b]Natural-log-transformed before analysis. [c]SBP + 10mmHg if on anti-hypertensive drug. [d]DBP + 5mmHg if on anti-hypertensive drug. [e]Hypertension in first degree relatives. BMI: Body mass index; CHD: coronary heart disease; DBP: diastolic blood pressure; DM: type 2 diabetes; F: Female; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment index of insulin resistance; HT: hypertension; LDL-C: low-density lipoprotein cholesterol; M: Male; n: Number; SBP: systolic blood pressure; TC: total cholesterol; TG: triglyceride.

Table 2. Genotype distributions and results of Cox-regression analyses.

SNP (1/2)		Genotype distribution Non-CHD (11/12/22)	Incident CHD (11/12/22)	MAF Non-CHD	Incident CHD	Unadjusted HR(95% CI)	P-value
rs1501299	**All**	**1103/759/148**	**88/75/19**	**0.262**	**0.310**	**1.26(1.01-1.56)**	**0.042**
(+276G>T)	**M**	**500/343/71**	**46/52/11**	**0.265**	**0.339**	**1.39(1.05-1.83)**	**0.020[a]**
(G/T)	F	603/416/77	42/23/8	0.260	0.267	1.04(0.73-1.50)	0.816
rs2241766	All	1007/822/183	89/83/12	0.295	0.290	0.97(0.78-1.22)	0.813
(T/G)	M	466/371/78	56/52/3	0.288	0.261	0.87(0.65-1.18)	0.374
	F	541/451/105	33/31/9	0.301	0.336	1.16(0.82-1.62)	0.401
rs12495941	All	680/966/362	56/95/32	0.421	0.434	1.05(0.86-1.29)	0.633
(G/T)	M	327/417/168	31/58/21	0.412	0.455	1.15(0.89-1.50)	0.278
	F	353/549/194	25/37/11	0.427	0.404	0.91(0.65-1.27)	0.592
rs822396	All	1511/468/30	137/46/1	0.131	0.130	0.99(0.73-1.35)	0.955
(A/C)	M	688/213/13	84/26/1	0.131	0.126	0.97(0.65-1.45)	0.878
	F	823/255/17	53/20/0	0.132	0.137	1.04(0.64-1.67)	0.884
rs822395	All	1441/527/41	130/53/1	0.151	0.149	0.98(0.73-1.31)	0.900
(A/C)	M	654/243/16	81/29/1	0.151	0.139	0.92(0.63-1.36)	0.674
	F	787/284/25	49/24/0	0.152	0.164	1.08(0.70-1.68)	0.720
rs182052	All	698/966/342	64/93/25	0.411	0.393	0.93(0.76-1.15)	0.510
(G/A)	M	319/436/157	41/53/15	0.411	0.381	0.89(0.68-1.17)	0.400
	F	379/530/185	23/40/10	0.411	0.411	1.00(0.72-1.39)	0.992
-10677C>T[b]	All	1755/248/8	156/25/3	0.066	0.084	1.26(0.87-1.83)	0.225
(C/T)	M	797/114/4	90/20/1	0.067	0.099	1.48(0.96-2.30)	0.078
	F	958/134/4	66/5/2	0.065	0.054	0.86(0.42-1.75)	0.671
rs266729	All	1148/729/130	111/65/8	0.246	0.220	0.88(0.69-1.12)	0.288
(C/G)	M	512/347/53	67/40/4	0.248	0.216	0.84(0.61-1.16)	0.298
	F	636/382/77	44/25/4	0.245	0.226	0.92(0.63-1.34)	0.653

rs16861194	All	1410/543/59	128/50/6	0.164	0.168	1.03(0.78-1.34)	0.854
(A/G)	M	642/248/25	80/28/3	0.162	0.153	0.94(0.65-1.35)	0.723
	F	768/295/34	48/22/3	0.165	0.191	1.17(0.78-1.76)	0.438

[a]Remained significant after adjustment for age and BMI. [b]No rs number is assigned for this SNP.

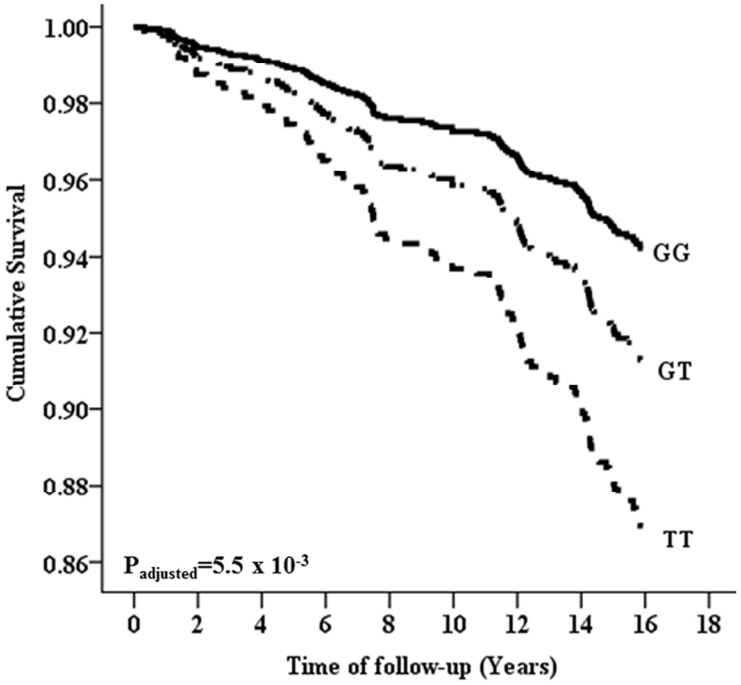
1: Major allele; 2: Minor allele; F: Female; M: Male; MAF: Minor allele frequency.

Table 3. Multiple Cox regression analyses of *ADIPOQ* +276 G>T and incident CHD in the male subjects.

Risk factors	Model 1		Model 2		Model 3	
	HR (95%CI)	P-value	HR (95%CI)	P-value	HR (95%CI)	P-value
+276G>T (T)	**1.54(1.13-2.08)**	**$5.5x10^{-3}$**	**1.44(1.08-1.93)**	**0.015**	**1.39(1.05-1.84)**	**0.023**
Age (years)	1.06(1.04-1.08)	<0.001	1.06(1.04-1.08)	<0.001	1.06(1.04-1.08)	<0.001
BMI (kg/m^2)	1.07(1.00-1.15)	0.044	1.08(1.02-1.15)	0.010	1.10(1.03-1.16)	0.002
DM	-	-	-	-	1.01(0.61-1.68)	0.976
FPG (mmol/l)	-	-	1.11(0.90-1.37)	0.331	-	-
2hrG (mmol/l)	1.00(0.95-1.05)	0.954	0.98(0.90-1.06)	0.562	-	-
HOMA-IR[a]	1.06(0.77-1.48)	0.711	-	-	-	-
Dyslipidemia	-	-	-	-	1.43(0.89-2.31)	0.143
HDL-C (mmol/l)	0.62(0.26-1.50)	0.292	0.57(0.24-1.32)	0.188	-	-
LDL-C (mmol/l)	0.91(0.71-1.17)	0.467	0.92(0.73-1.17)	0.504	-	-
TG (mmol/l)[a]	1.40(0.85-2.31)	0.187	1.40(0.86-2.28)	0.177	-	-
HT	-	-	-	-	2.03(1.31-3.14)	0.002
SBP[b] (mmHg)	1.01(0.99-1.03)	0.237	1.02(1.00-1.03)	0.088	-	-
DBP[b] (mmHg)	1.01(0.97-1.04)	0.744	1.00(0.97-1.03)	0.791	-	-
Smoking	1.25(0.96-1.88)	0.278	1.30(0.88-1.92)	0.183	1.82(0.88-1.91)	0.185

Model 1: Multiple adjustments made for age, BMI, 2hrG, HOMA-IR, HDL-C, LDL-C, TG, SBP, DBP and Smoking. Model 2: Multiple adjustments made for age, BMI, FPG, 2hrG, HDL-C, LDL-C, TG, SBP, DBP and Smoking. Model 3: Multiple adjustments made for age, BMI, DM, dyslipidemia, HT and smoking. [a]Natural-log-transformed before analysis. [b]SBP + 10mmHg and DBP + 5mmHg if on anti-hypertensive drug. 2hrG: 2hr post-OGTT glucose; BMI:

Body mass index; CI: Confidence interval; DM: type 2 diabetes; DBP: diastolic blood pressure; FPG: fasting plasma glucose; HOMA-IR: homeostasis model assessment index of insulin resistance; HR: Hazard ratio; HT: hypertension; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; SBP: systolic blood pressure; TG: triglyceride.



The cumulative survival curves for incident CHD in men, based on multiple Cox regression model 1, with adjustment for age, BMI, 2hrG, HOMA-IR, HDL-C, LDL-C, TG, SBP, DBP and smoking. The cumulative survival curves were stratified by the +276G>T genotypes.
61x46mm (600 x 600 DPI)

Supplementary table 1. Comparison of CRISPS2 adiponectin levels between different genotypes of +276G>T.

	+276G>T		
	GG	**GT**	**TT**
All (n=1676)	921	633	122
Adiponectin level (mg/l)	6.92(4.41-10.90)	6.64(4.28-10.08)	6.18(3.85-9.11)
Men (n=804)	433	307	64
Adiponectin level (mg/l)	5.89(3.59-9.47)	5.43(3.62-8.36)	4.93(3.62-7.19)
Women (n=872)	488	326	58
Adiponectin level (mg/l)	7.96(5.39-11.88)	7.93(5.39-11.75)	7.60(4.90-11.30)

Adiponectin levels at CRISPS2 were natural-log-transformed before analysis and data was presented as median with interquartile range.